Abnormal Lignins in Antisense-CCR and Antisense-CAD Plants

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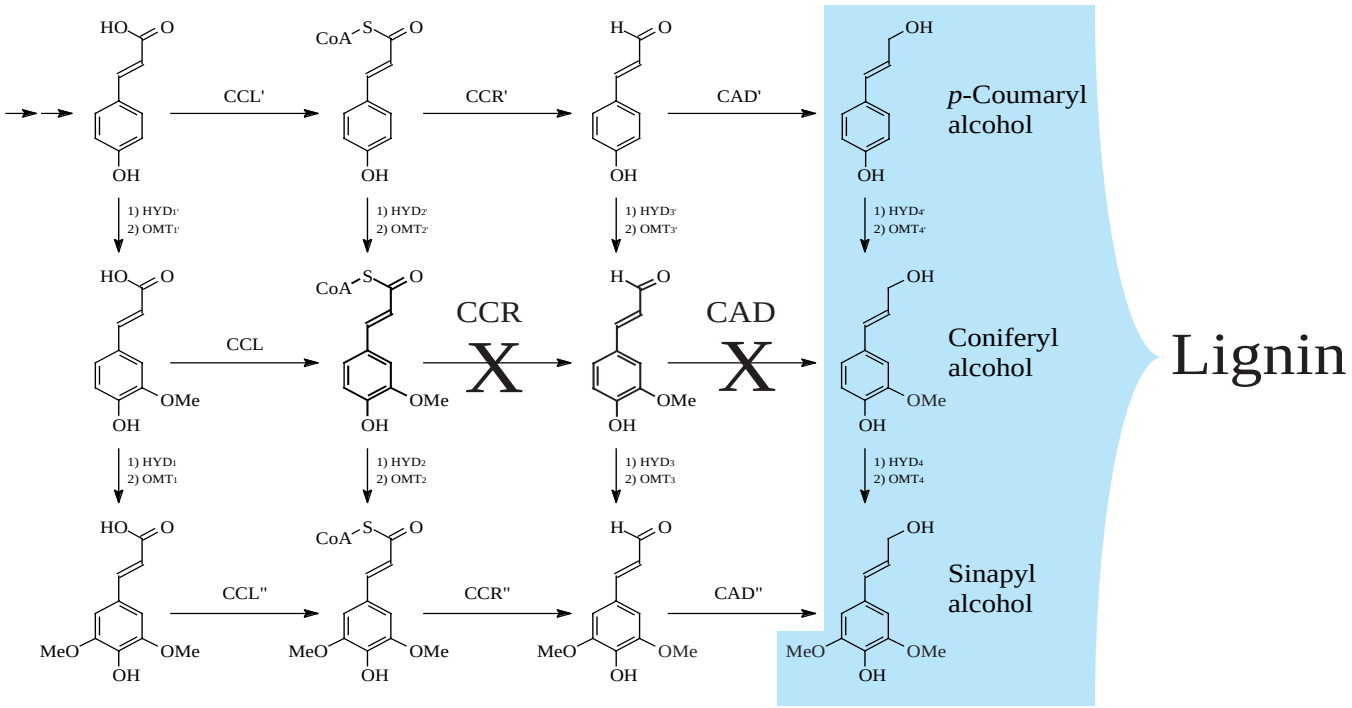
Introduction

Lignins are phenolic plant polymers essential for mechanical support, defense, and water transport in vascular terrestrial plants, but are a major obstacle to efficient utilization of plant polysaccharides in processes ranging from digestion in ruminant animals to industrial chemical pulping. Down-regulation of lignification is being actively pursued by many groups. A recent and promising approach has been the application of genetic biotechnologies targeting enzymes on the lignin monomer biosynthetic pathway, Scheme 1. Antisense applications are particularly attractive for research as they provide materials in which a single enzyme has been selectively targeted. Tobacco is an excellent model system due to the extensive literature on its analysis, chemistry, biochemistry, and the characterization of genes in the monolignol pathway. Antisense gene constructs were made to the CAD and CCR enzymes of tobacco by collaborators in France. For this study, lignins were isolated from the down-regulated CAD and CCR plants and compared to tobacco plants without the antisense constructs to examine how plants respond to the down-regulation of specifically targeted enzymes. This paper reports preliminary results obtained through the application of analytical NMR experiments.

Materials and Methods

Transgenic Tobacco Plants

(*Nicotiana tabacum* L. Cv. Samsun)



Scheme 1. Monolignol biosynthetic pathway. Not all of the enzymes are known or are necessarily discrete. Down-regulating CAD might be expected to build up coniferaldehyde; blocking CCR might build up feruloyl-SCoA, but then what?

Antisense CAD Plants. Seeds resulted from self pollination of primary transformant T37 carrying a 1kb CAD cDNA in antisense orientation, associated to the 35S CaMV promoter and the 3' terminator of the nopaline synthase.

Antisense CCR Plants. Seeds resulted from a test cross on primary transformant B3 carrying a 1.3 kb CCR cDNA in antisense orientation, associated to the 35S CaMV promoter and the 3' terminator of the nopaline synthase.

Both CAD and CCR antisense constructs also carried the neomycine phosphotransferase gene conferring resistance to kanamycin.

Lignin isolation and NMR were by normal procedures.

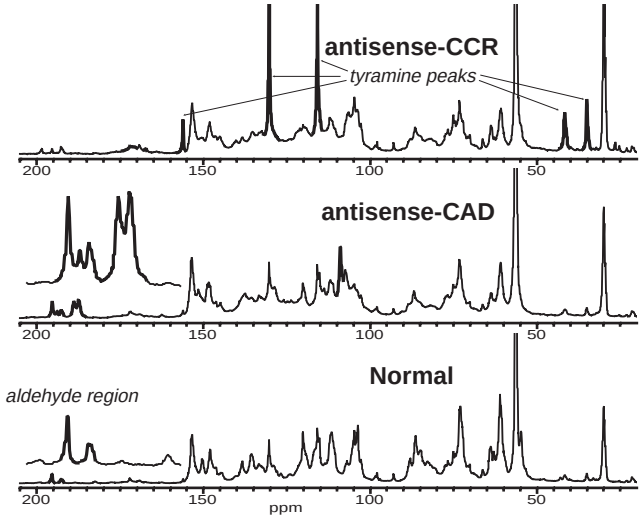


Figure 1. 13C NMR spectra of normal, antisense-CAD and antisense-CCR tobacco isolated lignins (unacetylated). The normal lignin is a syringyl/guaiacyl lignin, with guaiacyl units predominating. Aldehydes are elevated in the antisense-CAD sample; tyramine units (ex. tyramine ferulate) are markedly elevated in the antisense-CCR lignin.

Results and Discussion

Caveat

All following comments refer to the characterization of an extracted lignin. It is recognized that this is not the total lignin and may not be entirely representative of the in situ material; we will not continue to qualify each observation with that important point. However, isolation from the normal control and two antisense plants by identical procedures assures that differences noted are indicative of differences in the original plants. Additionally, the isolates characterized do have components that are not normally associated with lignin biosynthesis intimately incorporated by radical coupling processes with traditional lignin monomers into an analogous polymer. While this polymer is not a lignin by the traditional definition (...a polymer derived from three hydroxycinnamyl alcohol monomers...) it may have the properties to provide the required structural, water transport, and defense functions that the plant needs.

Antisense-CAD Tobacco Lignin

The Klason lignin level of the normal and antisense-CAD cell walls was similar (17% and 15%). Guaiacyl components (from coniferyl alcohol) were reduced as evidenced by the sharp reduction in traditional b-5 (phenylcoumaran) components visible in 2D experiments (not shown). It was the aldehyde region of the antisense-CAD lignin that displayed the most striking change, Fig. 1. Both cinnamaldehyde and benzaldehyde levels were higher (as expected from down-regulation of CAD) and new aldehydes became predominant. Benzaldehydes and cinnamaldehydes are well known components of lignins and are the agents responsible for the characteristic purple staining of lignified tissues by phloroglucinol-HCl. Whether these aldehydes are produced from normal hydroxycinnamyl alcohol sidechains by oxidative reactions following lignification, are from incorporation of monomers that have been oxidized to aldehydes by the oxidizing system, or are the result of direct incorporation of aldehydes that are exported to the wall before undergoing the final CAD-catalyzed reduction to the traditional alcohol monomers, is unknown. Direct incorporation of aldehyde monomers into free-radical polymerization reactions with normal monomers would explain their bonding patterns and relatively high contents, but other possibilities are not ruled out.

Aldehydes are assumed to be elevated components of CAD-deficient mutants, and some proof has been forthcoming, although distinguishing aldehydes that are

truly incorporated into the phenolic polymer from those present simply as extractives can be a problem. The red-brown coloration of CAD-deficient mutants has been ascribed to the presence of these aldehydes; synthetic lignins produced with coniferaldehyde incorporation have a similar red-brown color.

NMR indicates that the known benzaldehyde and cinnamaldehyde levels in the antisense-CAD isolated tobacco lignin are roughly doubled (Fig. 1). Extraction of the lignin after isolation with methylene chloride does not reduce this content — the aldehydes are an integral part of the polymer and are not simply un-extracted low molecular weight components associated with this fraction. Far more striking is the appearance of new aldehydes. They are at best barely discernible in the normal lignin but become major contributors to the antisense-CAD lignin (Fig. 1).

Antisense-CCR Tobacco Lignin

The Klason cell wall lignin content of this material was about half that of normal tobacco, but the extractable lignin was at about the same relative level. The stem material was quite flexible and plastic; unlike the more brittle normal and CAD-antisense tissues, it repeatedly lined around the grinder instead of following the normal path through the blades. The CCR mutant did not have normal development; plants severely depressed in CCR activity had a strong reduction in lignin content, reduced growth, abnormal leaf morphology, and collapsed vessels. Although its lignin content was lower, the percent of original lignin extractable was similar to the normal plant. The lignin showed clear evidence of the reduction of guaiacyl components, again by the lower levels of β-5 (phenylcoumaran) units, but the levels were not as drastically reduced as in the CAD-antisense plants. In this case, the syringyl components were relatively retained, as evidenced by the strong β-β-peaks in 2D spectra (not shown) and the $S_{2/6}$ carbons in the 1D ^{13}C NMR spectrum (Fig. 1).

Aldehyde levels were not significantly changed. The most striking change in this lignin was the dominance of tyramine units **Z** in the ^{13}C NMR spectra (Fig. 1). Huge *p*-hydroxyphenyl peaks were apparent that initially appeared attributable to *p*-coumarate or *p*-coumaryl alcohol units but were soon revealed to be tyramine-derived. They are totally free phenolic (within NMR sensitivity limits) as demonstrated by the expected shifts following acetylation of the phenol (not shown). As such they are reminiscent of *p*-coumarates in grasses. In both cases, the sharp, intense peaks result from a combination of the minuscule shift differences engendered by the moieties they are attached to, and the longer carbon relaxation times compared to main polymer carbons due to their being mobile endgroups. Other NMR experiments show that the tyramine is attached to ferulates. Tyramine ferulates are well known components in tobacco, and have been previously associated with the lignin. They are also associated with suberization, e.g., in wounded potatoes. Their levels increase with various stresses, including that produced by TMV infection. Interestingly, the levels were not notably different from the normal tobacco in the plant down-regulated by antisense-CAD methods, Fig. 1. Unfortunately, NMR spectra of tobacco lignins are sparse and other data is insufficient to determine whether the levels observed here are higher than would be observed in environmentally stressed plants.

With the above caveat that such units increase in stressed tobacco, tyramine ferulates are a logical sink for feruloyl–SCoA units that might be expected to build up when the CCR enzyme is down-regulated — CCR is the enzyme that takes hydroxycinnamoyl–SCoA's to cinnamaldehydes (Scheme 1). Tyramine derives from earlier in the pathway, from decarboxylation of tyrosine, a precursor of *p*-coumaric acid. The required transferase, hydroxycinnamoyl-coenzyme A:tyramine hydroxycinnamoyltransferase, has been found in many plants. It is obviously a leap to infer that the plant up-regulates production of tyramine specifically to provide a sink for feruloyl–SCoA and produce a derivative that can be incorporated into the lignification pathway to offset the deficit caused by decreased coniferyl alcohol. Nevertheless, it seems clear that, like the CAD-deficient mutant pine and the antisense-CAD tobacco, the antisense-CCR tobacco is making a copolymer that extracts as normal lignin and may serve many of the functions required of lignin

in the normal plant. It becomes a question of semantics whether or not to call this copolymer of tyramine ferulate and normal lignin monomers 'lignin,' but if it provides the plant with lignin's functionality, that seems appropriate. However, the CCR mutant does not have normal development. Clearly the functions required of lignin were not fully met by the reduced levels of this modified lignin. If the tyramine ferulate is intimately incorporated into the hydroxycinnamyl alcohol polymer, we have another example of the metabolic plasticity of plants to utilize other phenols to produce 'lignins' when normal monomers become limited. That the same units are used in certain stress and wounding responses perhaps indicates that this pathway can be up-regulated more quickly than the full lignin pathway to hydroxycinnamyl alcohols.

Conclusions

Down-regulating enzymes in the lignin monomer biosynthetic pathway may or may not reduce actual lignification. In the case of CCR, reduction was significant. In each down-regulated plant that has been structurally examined to date, the plant appears to compensate for its inability to produce (sufficient) normal lignin (lignin from the three hydroxycinnamyl alcohol monomers) by utilizing other phenols through up-regulation or redirection of other pathways. The materials isolated contain significant levels of new components. These 'lignin' isolations, performed equally for control and down-regulated plant samples, are relatively modest fractions of the total lignin, but clearly contain copolymers of normal lignin monomers and these new units. While we have good evidence regarding the structural claims for these modified lignins, their functional roles are not clear. The abnormality of heavily CCR down-regulated plants, which have 50% less lignin, does not clarify whether the plant's vigor is affected by lignin's quantity or its functional quality. Viability of mutants appears to be due to the plant's ability to produce lignin-like polymers with acceptable properties from unconventional components. Such a capability would appear to be evolutionarily wise and may be at least partly responsible for plants' abilities to remain viable despite mutations which severely curtail their normal biosynthetic pathways. If plants required and produced lignins exquisitely synthesized via highly controlled biochemical reactions, it would seem unlikely that they could, in a single generation, without the benefit of evolution, be viable when crucial components for that synthesis are heavily down-regulated. Although the plants' abilities to circumvent genetic obstacles by utilization of other components to make lignin interferes with our aims of significantly down-regulating lignification by genetic biotechnologies, it opens up enormous new potential for manipulation of lignin's composition and properties. Studies on lignin-biosynthetic-pathway-mutants will also provide a rich source of insight into the processes of lignification. In several instances now, down-regulation of the monolignol pathway has resulted in amplification of unusual units already present in low amounts in normal lignins.